The Thesis Committee for Margaret Drake-Studstill

Certifies that this is the approved version of the following Thesis:

The use of Rapamycin in reducing IL-10 and TGFβ expression in obesity-induced M2-like TAMs of the prostate tumor microenvironment

APPROVED BY

SUPERVISING COMMITTEE:

_____

Linda A. deGraffenried, Supervisor

_____

Christopher A. Jolly

The use of Rapamycin in reducing IL-10 and TGFβ expression in obesity-induced M2-like TAMs of the prostate tumor microenvironment

by

Margaret Drake-Studstill

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Abstract

The use of Rapamycin in reducing IL-10 and TGFβ expression in obesity-induced M2-like TAMs of the prostate tumor microenvironment

Margaret Drake-Studstill, MSNS
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Supervisor: Linda deGraffenried

Tumor-associated macrophages (TAMs) promote prostate cancer progression and aggressiveness in a variety of ways including promoting angiogenesis; upregulating tumoral survival; inducing EMT; and stimulating metastasis. Most importantly, they can suppress the immune system by expressing anti-inflammatory cytokines such as IL-10 and TGFβ. M2-like TAMs are polarized by a variety of signals, including from signaling factors expressed by adipocytes in the case of obese patients. It is crucial that we find ways to reduce immune suppression in cancer patients, so that patients' immune systems may slow the cancer progression.

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Chapter 1

Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer in men and it is estimated that one out of every nine men will be diagnosed in their lifetime. In 2018 alone, there were over 164,000 new diagnoses and almost 30,000 deaths from PCa (Society, 2018). Whether a patient receives treatment or is placed under surveillance depends on disease stage at the time of detection. Typically, patients whose cancer is detected early and is accompanied by slow growth are placed under surveillance. Treatment can be local, systemic or both. Local treatment options include surgical prostatectomy and radiation therapy, both carrying the risk of permanent erectile dysfunctional and chronic incontinence. Systemic treatment options include androgen deprivation therapy (ADT) and chemotherapy, both carrying the risk of an array of undesirable side effects beyond those affecting the genitourinary system. Up to half of all PCa patients may be over-treated, leading to permanent and unnecessary side effects (Hoffman et al., 2014; Loeb et al., 2014). Identification of reliable biomarkers are required to distinguish indolent versus aggressive cases of PCa, which could reduce over-treatment of patients who have a low risk of progression (Evans et al., 2009; Spahn et al., 2015). Along with utilizing reliable biomarkers, treatment with immunotherapy could reduce the risk of toxic permanent side effects associated with the older treatment options.

ADT, an older systemic treatment option, works by reducing circulating testosterone in hormone dependent PCa. ADT comes with the additional risk of promoting progression to a more treatment resistant, metastatic cancer (Mukherji, Omlin, Pezaro, Shamseddine, & de Bono, 2014). Castration-resistant prostate cancer (CRPC) occurs when PCa becomes hormone-independent in

response to prolonged ADT, allowing the cancer to metastasize, often to the bone, and become chemo-resistant (Allott, Masko, & Freedland, 2013; Suarez et al., 2014).

Inflammation is a well-studied factor in cancer progression and plays a role in tumorigenesis through a variety of mechanisms including modulation of immune cells and cytokine signaling pathways (Grivennikov, Greten, & Karin, 2010). Sources of inflammation include diet quality and obesity status, both of which are linked to disease aggressiveness and patient prognosis (Allott & Hursting, 2015; Moller et al., 2015). Chronic low-grade inflammation affects not only the PCa epithelial cells, but also the fibroblasts, blood vessels, immune cells, adipocytes, stromal cells and endothelial cells of the tumor microenvironment (TME) (Chiarugi, Paoli, & Cirri, 2014; Zeigler-Johnson, Morales, Lal, & Feldman, 2016). Tumor-associated macrophages (TAMs) of the TME are a particularly interesting immune phenomenon influenced by cancer and may have potential as prognostic biomarkers or as therapeutic targets.

While traditional treatment focuses on targeting cancer cells themselves, recent research has focused on training immune cells of the TME to inhibit cancer cell growth. TAMs are a viable target to improve PCa prognosis due to the multitude of mechanisms by which they influence the TME. TAM presence in the TME is typically associated with a worse prognosis and increased rates of metastasis (Quail & Joyce, 2013; Seyfried & Huysentruyt, 2013). Targeting the TAMs themselves or their specific pro-tumorigenic, pro-angiogenic, or anti-inflammatory actions can be a viable step to improve patient outcome.

FROM MACROPHAGES TO TUMOR-ASSOCIATED MACROPHAGES

Macrophages are innate immune cells derived from hematopoietic stem cell progenitors (HSCs) of the bone marrow (Cortez-Retamozo et al., 2012). HSCs continuously proliferate and shed their progeny into the bloodstream as promonocytes, which develop into monocytes and extravasate into tissues exhibiting low-grade inflammation, such as neoplastic tissue (Lewis & Pollard, 2006; Mallat, 2014). Once in the tissue, monocytes undergo differentiation into macrophages of varying phenotypes (Cortez-Retamozo et al., 2012). Macrophage polarization is dictated by environmental stimuli, and it is known to be a reversible process. Of the macrophage subtypes, the two most commonly studied lie on opposite ends of the polarization spectrum: M1 and M2 macrophages (Lanciotti et al., 2014).

M1, or classically activated, macrophages predominantly display cytotoxic and pro-inflammatory effects in the TME, while M2 macrophages are anti-inflammatory and tumor promoting (Lanciotti et al., 2014). M1 macrophages are characterized by high secretion of IFN-γ, IL-1, IL-6, IL-12, IL-23, and TNF-α and cell surface expression of CD80 and CD86. M2 macrophages are characterized by the high secretion of IL-10, TGF-β, IL-8/CXCL8, VEGF, and cell surface expression of CD68, CD163 and CD206 (Mills, 2015). Pro-tumorigenic TAMs of the TME typically display cytokine and cell surface profiles like that of the M2 polarized macrophages (Y. Liu & Cao, 2015; Rhee, 2016).

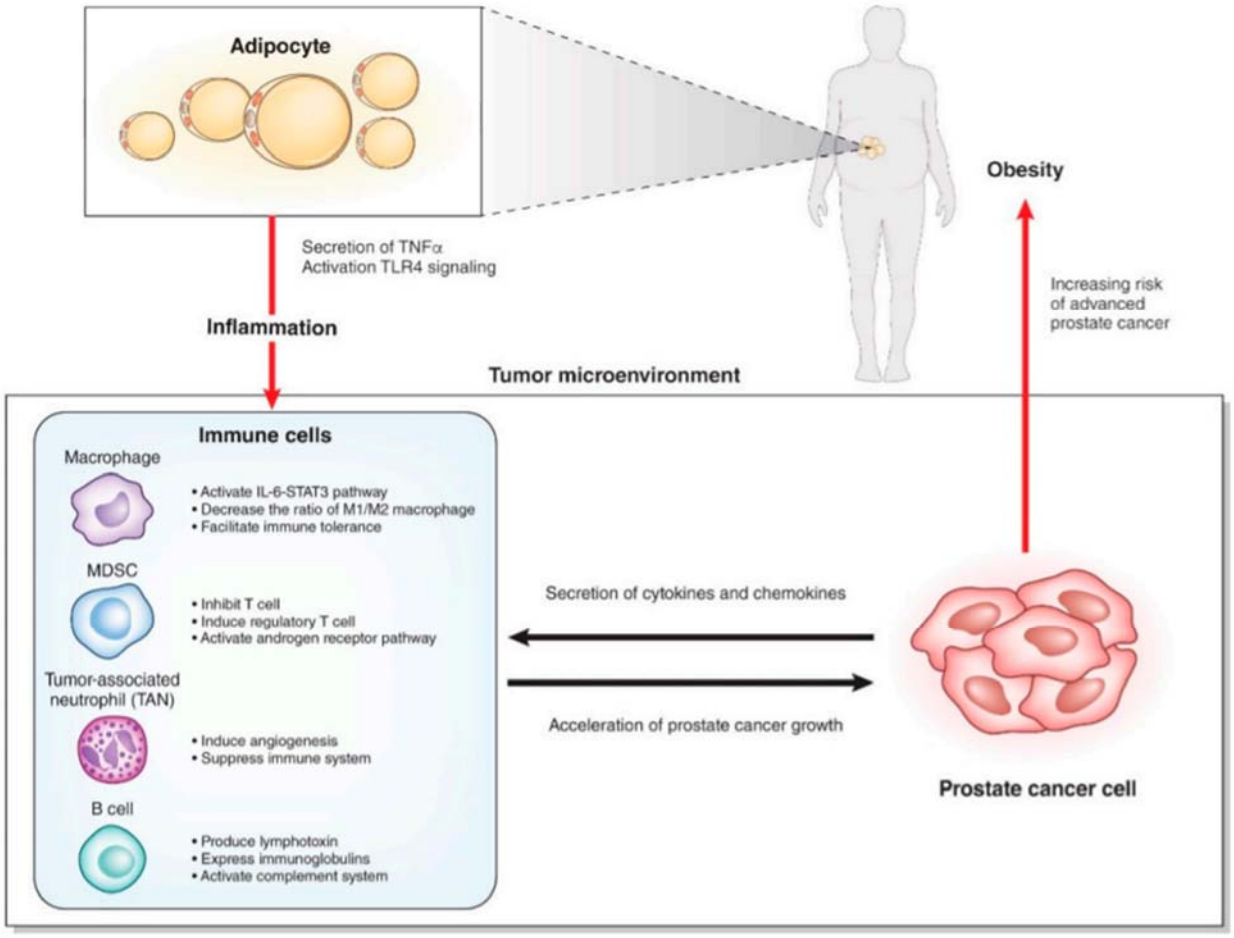
Macrophages are recruited to the TME by a variety of signals, including colony-stimulating factor-1 (CSF-1), CC ligand 2 (CCL2), and stromal-derived factor-1 (SDF-1, also known as CXCL12). CSF-1 is highly expressed by epithelial cancer cells, and its receptor (CSF1R) is expressed on macrophages and monocytes (Cannarile et al., 2017). CCL2 is a cytokine that mediates monocyte recruitment via interaction with the chemokine receptor CCR2 (Sierra-Filardi

et al., 2014). SDF-1 is upregulated in damaged tissue to recruit macrophages via interaction with CXCR4 (Sanchez-Martin et al., 2011).

Macrophages are polarized according to the different signals they receive in the form of cytokines and chemokines. IFN-γ produced by Th1 cells tends to promote polarization into M1 macrophages. IL-4 and IL-13 produced by Th2 cells tends to promote polarization into M2 macrophages. Tumor cells themselves are known to participate in the recruitment, differentiation and polarization of macrophages. Comito, et al., in 2014, reported that PCa cells secrete CCL2 to recruit monocytes and subsequently influence differentiation and polarization into M2 or M2-like TAM phenotypes (Comito et al., 2014).

As obesity incidence is increasing, it is important to understand how obesity affects cells of the immune system, including macrophages. Insulin resistance and chronic adipose inflammation frequently occur alongside obesity, and together they affect how macrophages are polarized. Adipose tissue releases MCP-1, as well as IL-6, MIP-α, and CSF-1 (Gesta, Tseng, & Kahn, 2007). Adipocytes of the visceral adipose tissue die at a higher rate than those of subcutaneous fat, and this hypoxia-induced cell death recruits macrophages and polarizes them to the M1 phenotype (Cinti et al., 2005). The number of macrophages present, specially M1 macrophages, is associated with insulin resistance (Kanda et al., 2006). These M1 macrophages frequently aggregate into “crown-like” structures around the inflamed adipocytes (Lumeng, DelProposto, Westcott, & Saltiel, 2008). While M1 macrophage accumulation is a problem in non-cancerous obese individuals, when cancer is added to the mix, the reverse is true and alternatively activated macrophages become the problem. A yet-identified cytokine or chemokine produced by adipocytes (possibly leptin, IGF-1, or IL-6) interacts with cancer cells to trigger their production of another yet-identified cytokine or chemokine that preferentially polarizes macrophages towards

the M2-like TAM phenotype (Galvan et al., 2017). This is an effect seen at higher rates in obesity, and results in greater immune suppression.



(Fujita, Hayashi, Matsushita, Uemura, & Nonomura, 2019)

MECHANISMS BY WHICH TAMS MODULATE THE TME

Following macrophage recruitment and polarization to the M2 phenotype, TAMs can modulate the TME to promote cancer progression. TAMs typically accumulate in poorly vascularized and necrotic areas (Coffelt, Hughes, & Lewis, 2009). TAMs promote cancer progression by secreting anti-inflammatory cytokines to create an immunosuppressive TME and enhance tumor angiogenesis, survival and metastasis (Hanahan & Coussens, 2012; Soave et al., 2016). Angiogenesis is the process by which new vessels in the TME are formed to support tumor growth by increasing oxygen and nutrient availability (Hanahan & Weinberg, 2011). TAMs contribute to angiogenesis by secreting a variety of pro-angiogenic mediators, such as: basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator (uPA), adrenomedullin (ADM), VEGF via the NF-κB signaling pathway, IL-6, IL-10, HIF-1, and HIF-2 (Chanmee, Ontong, Konno, & Itano, 2014; Rhee, 2016). Human clinical studies have shown an association between high TAM concentration, increased microvessel density indicating effective angiogenesis, and poor prognosis in a variety of cancers, including prostate (Kurahara et al., 2011; C. Li, Shintani, Terakado, Nakashiro, & Hamakawa, 2002; Ohno et al., 2004; Onita et al., 2002; Takanami, Takeuchi, & Kodaira, 1999).

Another important hallmark of cancer progression is tumor survival, or the ability to resist apoptotic triggering of cell death (Hanahan & Weinberg, 2011). TAMs function under a variety of mechanisms to promote increased tumoral survival. One mechanism is the release of IL-1β, which inactivates GSK3β, enhances Wnt signaling, and confers a resistance to TRAIL-induced apoptosis (Kaler, Galea, Augenlicht, & Klampfer, 2010). A second mechanism is through increased production of IL-6 which phosphorylates the transcription factor STAT3 which upregulates expression of tumor growth factor (TGF-β1) and hypoxia-inducible factor (HIF-1α) (De Beule et

al., 2017; Jeong et al., 2017; Zhou et al., 2015). TGF-β1 is a regulatory cytokine that activates FosB to promote tumor survival, migration and invasion through induction of transcription of pro-survival genes (Barrett, Millena, & Khan, 2017). HIF-1α is a transcription factor that becomes activated in low oxygen conditions to transcribe pro-survival genes (Thomas & Kim, 2008).

Interestingly, TAM production of IL-6 and the subsequent STAT3-induced expression of TGF-β1 has also been implicated in the expansion of CD44 cancer stem cells in the TME and the induction of the epithelial to mesenchymal transition (EMT) (Q. M. Fan et al., 2014; Wan et al., 2014). Cancer cell EMT to a more stem-like phenotype confers worse outcomes, as epithelial cancer cells gain invasive and metastatic capacities while losing cell-to-cell contact (Singh & Settleman, 2010).

TAMs stimulate metastasis through mechanisms such as tissue remodeling and altering the extracellular matrix (ECM) structure. Some *in vitro* studies have found that metastasis and invasion is promoted by TAMs through their release of metalloproteinases (MMPs), such as MMP-9 and MMP-2, and their secretion of epidermal growth factor (EGF) (Goswami et al., 2005; Kessenbrock, Plaks, & Werb, 2010). MMPs are endopeptidases associated directly with tissue remodeling and ECM alteration (Y. Gong, Chippada-Venkata, & Oh, 2014). EGF is a chemotactic factor that operates through EGF receptor (EGFR) to accelerate cancer cell metastasis, particularly to the bone (Chang et al., 2015). *In vivo* PCa models have shown that macrophage depletion therapy and anti-CCL2 antibodies reduce macrophage infiltration in tumors and reduce metastasis to the bone and lymph nodes (S. W. Kim et al., 2011; Loberg et al., 2007). Furthermore, accelerated metastasis has been observed in tumors that overexpress the macrophage recruiter CSF-1 (Lin, Nguyen, Russell, & Pollard, 2001).

In summary, TAMs are known to promote cancer progression and aggressiveness by promoting angiogenesis, upregulating tumoral survival, inducing EMT, and stimulating metastasis. In theory, the higher the TAM concentration in the TME, the worse the prognosis, hence their concentration as potential biomarker to predict disease progression.

TAMS AND PROSTATE TUMOR GRADE

Current standards evaluate PCa stage at the time of diagnosis using a prostate specific antigen test (PSA) the Gleason scoring system (Kattan, 2007). The minimally invasive PSA test is used to detect the possible presence of PCa in an individual. PSA levels are obtained by routine blood test, whereas Gleason scores are obtained after a patient has undergone a biopsy. Biopsies usually occur after an elevated PSA level, which is above 4.0 ng/mL. The PSA test measures prostate-specific androgen levels, a protein produced by cells of the prostate gland. A patient's PSA level can be elevated for non-cancerous reasons, such as benign prostatic hyperplasia (BPH), urinary tract infections, sexual intercourse, medical procedures, rectal exams, and age, and thus PSA is not a reliable biomarker and oftentimes reliance on PSA values results in an unnecessary biopsy.

Gleason scores range from one to nine and are grouped into three grades – low, medium and high. Each section of a prostate biopsy receives a score of one to nine based on the severity of cancer in that section, and the scores are averaged to calculate an overall Gleason score (Epstein, 2010). While Gleason scores are still used in clinical settings, a new scoring system for PCa called the 5 Grade Group system has been developed to more accurately stratify PCa grades and simplify scores to avoid patient confusion. However, most practicing physicians are accustomed to using Gleason scores and not all are trained to use the 5 Grade Group system. Additionally, when the 5 Grade Group system was introduced, recommendations called for giving patients both their Gleason score and their 5 Grade Group score, thereby exacerbating the confusion the new scoring system hoped to circumvent (N. Chen & Zhou, 2016). Hence, Gleason scores have been allowed to persist clinically, and oftentimes, solely.

While low-grade Gleason scores (1-3) warrant active surveillance consisting of biannual PSA tests, and high-grade scores (7-9) typically require prostatectomy, the treatment route for medium-grade scores (4-6) is dictated by interpretations by individual clinicians and the choices of their patients. Clinicians and patients typically chose to treat medium-grade PCa tumors aggressively, but since there is no reliable biomarker to predict whether a medium-grade score PCa tumor will progress or stagnate, the likelihood of unnecessary overtreatment is high. Unnecessary overtreatment is problematic because aggressive treatment of stagnating, medium-grade prostate cancers may be more detrimental to the patient's health than living with the cancer. There is a substantial need to find a reliable prognostic biomarker to reduce the overtreatment of PCa, especially for patients with medium-grade tumors.

CURRENT APPROACHES TARGETING TAMS

As the non-cancerous cells of the TME, such as TAMs, can enable multiple mechanisms that enhance tumor progression, targeting the TME rather than just the cancerous cells is an effective strategy for treating cancer (Hanahan & Coussens, 2012). Suppressing the pro-tumorigenic activities of cells in the TME can be used in conjunction with chemotherapy, prostatectomy, radiation therapy, ADT or immunotherapy to synergistically improve response to treatment and prevent recurrence. Targeting the cytokines and chemokines that promote macrophage recruitment and polarization into the M2-like TAM phenotype can block the initial infiltration and polarization into pro-tumorigenic TAMs. Many current studies focus on blocking this recruitment and subsequent polarization, but far fewer studies have examined the possible reprogramming of TAM-like M2 macrophages back to the anti-tumorigenic, pro-inflammatory M1 phenotype (Heusinkveld et al., 2011).

BLOCKING MACROPHAGE RECRUITMENT TO THE TME

Many current therapeutic interventions focused on TAMs aim to block macrophage recruitment and infiltration into the TME, thereby decreasing the availability of macrophages to become polarized towards a M2-like TAM phenotype under the influence of the prostate cancer cells. Inhibitors of CCL2 and SDF-1 or their macrophage receptors CCR2 and CXCR4 have been implicated in blocking TAM recruitment and blunting their influence on the TME (Gupta & Duda, 2016; Lim, Yuzhalin, Gordon-Weeks, & Muschel, 2016; Loberg et al., 2007). One study demonstrated that CCL2 neutralizing antibodies slowed prostate cancer cell growth and metastasis by blocking macrophage recruitment to the TME (Mizutani et al., 2009). Omega-3 fatty acid supplementation in prostate cancer patients has been shown to reduce CCL2 expression, thereby affecting macrophage recruitment and resulting in significantly reduced tumor volume compared

to a control group (Liang et al., 2016). A later study connected high CCL2 expression as an effect of increased Wnt signaling, specifically that of WNT5A, suggesting that WNT5A could be a more specific therapeutic target to reduce macrophage recruitment to the TME as well as reduce the development of CRPC and metastasis to the bone (Lee et al., 2018).

Other therapeutic strategies focused on reducing macrophage infiltration target CSF1 to block the CSF1-CSF1R interaction thus inhibiting macrophage recruitment, proliferation and survival. A combination treatment including CSF1-CSF1R inhibitors and ADT results in decreased TAM recruitment, repressed tumorigenesis and delayed tumor regrowth in mice compared to ADT alone (Escamilla et al., 2015). Guan, et al., in a study published in 2019, found that supplementing docetaxel, a chemotherapeutic agent, with an inhibitor of CSF-1 receptor kinase, suppresses tumor growth better than docetaxel alone, in part because of the decreased TAM infiltration (Guan et al., 2019).

Pigment Epithelium-Derived Factor (PEDF) has recently been found to aid in macrophage recruitment and polarization into M1, presenting another possible target to reduce macrophage recruitment or possibly a tool to repolarize TAMs to M1 macrophages (Nelius et al., 2013). While it has not yet been elucidated if PEDF can repolarize TAMs to M1 macrophages, PEDF has been found to increase phagocytosis of PCa cells by macrophages, suggesting either preferential infiltration of M1 macrophages or promotion of polarization towards a more M1-like phenotype (Martinez-Marin et al., 2017).

Protein kinase C (PKC) isozymes have also been recently investigated for their role in regulating macrophage recruitment and polarization in the TME. Reduced PKCζ expression in PCa has been found to promote macrophage recruitment to the TME and subsequent polarization towards the M2-like TAM phenotype mediated through IL-4 and IL-10 (Fan et al., 2017).

Expression of PKCζ is inversely associated with cancer stage and the concentration of M2-like CD206+ TAMs. This discovery indicates that PKCζ should be considered a tumor suppressor and that blocking both IL-4 and IL-10 could reduce TAM presence in the TME.

Metformin is currently being investigated for its potential to block macrophage recruitment to the TME in PCa. Liu et al recently elucidated the mechanism by which metformin blocks macrophage recruitment, finding that metformin downregulates the COX2/PGE2 axis, directly reducing the number of TAMs recruited to the TME and thus slowing tumor growth (Q. Liu et al., 2018). Wang, et al., in a study published in 2017, found that enzalutamide resistance, such as that seen in CRPC, occurs in part from a feedback loop whereby TAMs are recruited to the TME, and can be attenuated by inhibiting IL-6R and high mobility group box 1 (HMGB1) (C. Wang et al., 2018). A year later, Huang, et al., confirmed that inhibiting IL-6R ameliorates ADT (such as enzalutamide) resistance, such as that seen in CRPC (Huang et al., 2018). Research by Yeh et al, also identified IL-6 as an important target to reduce M2 macrophages (Yeh et al., 2016). Liu et al., also in 2017, confirmed that metformin is capable of inhibiting IL-6R, thereby blocking TAM recruitment to the TME and overcoming enzalutamide resistance (Q. Liu et al., 2017). Metformin as a combination treatment with enzalutamide for prostate cancer is currently in clinical trial, though the combination is studied more for the metabolic effects than the recently discovered immunomodulatory effects (Institute, 2014).

BLOCKING POLARIZATION OF MACROPHAGES TO M2-LIKE PHENOTYPE

As it has been established that downregulating the COX2/PGE2 axis slows tumor growth, additional research has investigated the additional mechanisms between this cause and effect (Q. Liu et al., 2018). While Liu et al showed that the reduced tumor growth was due to a reduction in macrophage recruitment to the TME, other research suggests that downregulating the COX2/PGE2

axis blocks macrophage polarization towards a M2-like TAM phenotype. Non-steroidal anti-inflammatory drugs (NSAIDs) have been proposed as a potential therapy to block polarization towards a M2-like TAM phenotype given that they can inhibit prostaglandins involved in macrophage polarization via inhibition of the cyclooxygenase-2 (COX-2) pathway (Pollard, 2004).

A unique body of research combining molecular oncology and nutritional sciences focuses on macrophage polarization as it is affected by glycation products, which are proteins or fats with modified sugar moieties ubiquitous in food common in the Western diet. Advanced glycation end-products (AGEs) are thought to promote cancer progression, including that of the prostate. Early glycation products (EGPs) are less studied, but evidence is confirming that they play a role in promoting cancer progression as well. Chen, et al., in two studies published in 2018, found that AGEs directly promote PCa progression, but that EGPs indirectly promote PCa progression by assisting PCa-induced polarization towards the M2 phenotype (Y. Chen, Filipov, & Guo, 2018; Y. Chen & Guo, 2018). Clinical trials are already underway investigating the combined effects of metformin and oligomeric procyanidin complex on reducing glycation products and M2 polarized macrophages in PCa patients receiving ADT (Instiute, 2016).

A recent study by Liu, et al., 2019, uncovered a novel possible way to block polarization towards the M2-like TAM phenotype (Z. Z. Liu et al., 2019). TRIB1 is a pseudokinase that plays a non-specific role in many of the pathways involved in cellular differentiation and tumor angiogenesis. Liu, et al., found that in PCa cells, TRIB1 inhibits IKB-zeta of the NFkB transcription factor pathway, reducing the secretion of cytokines that direct polarization towards the M2-like TAM phenotype. A small molecule mimetic of TRIB1 targeted to PCa cells could be developed as an immunotherapy adjuvant to reduce M2-like TAM polarization.

TARGETING M2 SPECIFICALLY

Zoledronic acid (ZA), an osteoporosis drug that is also prescribed for bone metastasis pain, is currently being investigated for its use in preferentially targeting M2 or TAM-like macrophages. ZA has been shown in breast cancer cells to reduce VEGF in the TME which subsequently repolarizes TAMs towards an M1-like phenotype (Coscia et al., 2010). A study by Comito, et al., in 2017, sought to examine the effect ZA has on immunomodulation in a PCa model. They reported that ZA treatment reduced expression of anti-inflammatory IL-10, reversed M2 polarization and had no effect on M1 macrophages (Comito et al., 2017). While ZA needs further research to determine viability as a treatment adjuvant, the preliminary results indicate its potential as a powerful immunomodulation agent.

A recent study has focused on Tyro3, Axl and MerTK, TAM receptors that could serve as therapeutic targets (Myers, Amend, & Pienta, 2019). These receptors, along with the ligands Gas6 and Protein S, aid in macrophage polarization. Tyro3, Axl and MerTK are also receptors for the pro-tumorigenic process of efferocytosis, when they become liganded by phosphatidylserine on apoptotic cells and the macrophage subsequently engulfs and clears the apoptotic cell. Efferocytosis is pro-tumorigenic because it suppresses the immune system to reduce inflammation, which in turn further polarizes macrophages to the M2 or TAM-like phenotype. Interfering with TAM receptor signaling could both block a pro-tumorigenic effect of TAMs as well as reduce subsequent polarization towards the TAM-like phenotype. R428, an inhibitor of Axl, has been shown to increase sensitivity to the anti-proliferative effects of metformin in LNCaP prostate cancer cells previously conferring metformin resistance (Bansal, Mishra, Stein, DiPaola, & Bertino, 2015). The findings by Myers, et al., are in line with an earlier study by Yin, et al, that focused on the phosphatidylserine ligand rather than the TAM receptors themselves (Yin, Huang, Lynn, & Thorpe, 2013). In their 2013 study, they found that phosphatidylserine antibody 2aG4

administered along with docetaxel reduced TAM presence and increased M1 macrophages in the prostate cancer TME.

TARGETING PRO-ANGIOGENIC FACTORS AND ANTI-INFLAMMATORY CYTOKINES PRODUCED BY TAMS

Several studies focus on targeting the different pro-angiogenic factors and anti-inflammatory cytokines and proteins secreted by or displayed on TAMs. Tasquinimod, a small-molecule inhibitor in phase III development, targets the PCa TME by blocking immunosuppressive, pro-metastatic signaling from TAMs (Olsson et al., 2015; Shen et al., 2015). Clinical trials have found that tasquinimod increases survival time compared to placebo and in patients with bone metastases (Armstrong et al., 2013). Other therapies have targeted the pro-angiogenic factors expressed by TAMs, such as VEGF, though their effectiveness is short-term as tumors can develop resistance (Casanovas, Hicklin, Bergers, & Hanahan, 2005).

A recent study focused on the effect IL-8 secreted by M2-like macrophages has on the MALAT1 expression in PCa (Zheng, Ma, Tang, Li, & Xu, 2018). MALAT1 is a long, non-coding RNA oncogene overexpressed in many cancer types, including prostate cancer, and has recently been associated with PCa prognosis and identified as a therapeutic target (Cao et al., 2016; Y. Fan et al., 2014; Kwok, Roche, Chew, Fadieieva, & Tay, 2018; Ren et al., 2013; F. Wang et al., 2014). Zheng, et al, 2018 found that the IL-8 secreted by M2 macrophages activates STAT3 to bind to MALAT1's promoter and increase expression of the RNA oncogene, leading to PCa tumor progression. Their study identified several possible points at which new immunotherapy could be developed to block the effect M2 macrophages have on tumor progression.

MicroRNAs are another field receiving attention for their roles in regulating the effects of TAMs. MicroRNAs are non-coding RNA molecules around 20 nucleotides long that post-transcriptionally modify coding RNA transcripts. miR-124, -142-5p, -146a and -511 have been identified as promoting polarization towards a M2-like TAM phenotype, which miR-17-5p and -34a seem to inhibit polarization towards a M2-like TAMs phenotype (Self-Fordham, Naqvi, Uttamani, Kulkarni, & Nares, 2017). A recent study identified Let-7b, a miRNA with a role in proliferation and inflammation, as a possible target to modulate the anti-inflammatory cytokine profiles of PCa-induced TAMs. A decrease in expression of or the use of inhibitors to Let-7b is associated with inhibition of pro-angiogenesis of TAMs and an inhibition of PCa cell motility (Z. Wang et al., 2016). Again, additional research is required, but miRNAs, specifically those of the Let-7 family, may be useful targets to attenuate the pro-angiogenic and anti-inflammatory effects of TAMs, which other miRNAs may be useful in regulating the polarization plasticity of macrophages.

TAM-targeted therapies, including blocking TAM recruitment to tumors, preventing the polarization of macrophages into the pro-tumorigenic phenotype, inducing the reprogramming of TAMs or limiting the function of TAMs have been shown to have potential in slowing cancer progression (Ruffell, Affara, & Coussens, 2012). An enormous benefit of macrophage-targeted therapy is the reduced risk of drug resistance because they have stable genomes without malignant mutations (Condeelis & Pollard, 2006). In addition to TAMs, other non-cancerous cells that interact with or support TAMs in the TME, such as cancer-associated fibroblasts (CAFs) and T cells, should be further evaluated as potential therapeutic targets. Combination therapies could then be developed to create a synergistic and more efficacious treatment.

CURRENT EVIDENCE TO THE BIOMARKER POTENTIAL OF TAMS

Due to the *in vitro* studies associating TAMs with angiogenesis, metastasis and tumor survival, TAM concentration represents a promising potential biomarker for cancer progression or predicting recurrence. Early studies using immunohistology in prostatectomy sample found that there is an association between TAMs and Gleason score and that TAM concentration can predict time to disease progression after prostatectomy (Shimura et al., 2000). A later study found that TAM concentration can also predict cancer progression after ADT (Nonomura et al., 2011). A 2013 study by Gollapudi, et al., conflicted the earlier findings from Shimura, et al., 2000. Their study using a tissue microarray of 332 patients found that while average TAM number was higher in patients with Gleason scores of 4 than in patients with Gleason scores of 3, there was no association between TAMs and biochemical recurrence or time to disease progression after prostatectomy (Gollapudi et al., 2013). The conflicting studies on recurrence prediction after surgery indicate that further evaluation is required. Studies still also must be conducted evaluating TAM concentration as a predictor of disease progression specifically in mid-range Gleason score patients prior to undergoing treatment.

Mouse xenograft models have been useful to determine an association between tumor growth and M2-polarized TAM concentration. In mice xenograft models, human prostate cancer cells such as LAPC4, PC3, DU145 and LNCaP are implanted subcutaneously into mice and allowed to grow into tumors, which are then excised and analyzed, along with other components of the tumor microenvironment, such as lymph nodes and ascites. A recent study by Copeland, et al., found that tumors that grew at the fastest rate were accompanied by the greatest concentration of M2-polarized TAMs, suggesting that M2-polarized TAM concentration, as measured by CD68 and CD206, could we useful in predicting tumor growth rate (Copeland et al., 2019). They also

found that nearly all (94%+) of macrophages in the excised sections were polarized to an M2-like phenotype, further confirming that cancer cells drive polarization preferentially towards the M2-like phenotype.

A 2015 study by Ok Atilgan, et al., postulated that TAM concentration in conjunction with measured expression levels of certain related regulatory proteins could serve as a useful prognostic panel (Ok Atilgan et al., 2016). In their study, samples from 100 prostate cancer patients were retrospectively examined for TAMs, Hexim1, SMADs and TGFβ. SMADs are cytoplasmic signal transducers whose transcription is regulated in part by TGFβ, and Hexim1 regulates TGFβ signaling pathways. They found that while TAM concentration was correlated with Gleason score and cancer stage, TAM concentration in conjunction with strong expression of Hexim1 and SMAD2 and weak expression of SMAD7 better predicted disease progression. Ok Atilgan, et al., also proposed therapeutic target potential of the SMADs and Hexim1.

Conversely, rather than focusing on the concentration of TAMs as a biomarker of disease progression, some studies have focused on the concentration of M1-like macrophages as a biomarker of better prognosis (Adamo et al., 2019; Zhang et al., 2019). This is a unique perspective considering the assumption made under strong evidence that cancer cells preferentially polarize macrophages towards the M2-like phenotype. The study by Adamo, et al., theorized that glandular epithelial cells surrounding tumors is as capable of polarizing macrophages as the tumor cells themselves. Rather than using a xenograph model, their study focused on analyzing expression patterns of the C/EBPβ transcription factor in both rat and human prostate cancer samples. They found that increased C/EBPβ transcription factor expression in tumor cells is associated with poor outcome, but that increased C/EBPβ transcription factor expression in epithelial cells surrounding the tumor is associated with better outcome due in part to polarizing macrophages in the TME to

an immune-stimulating M1-like phenotype. Adamo, et al., proposed that measuring expression levels of C/EBPβ transcription factor in the glandular epithelial cells could predict the presence of an aggressive tumor with metastatic potential.

Using CIBERSORT, an analytical tool measuring cell type abundance, Zhang, et al., corroborated the discovery that high M1 macrophage presence in the TME is associated with better prognosis, as shown by Adamo, et al. For their study, Zhang, et al., used samples from healthy prostate tissue and cancerous prostate tissue from patients with clinical follow-up data. They found that PCa patients with high M1-like macrophages had improved overall survival compared to patients with lower M1-like macrophage concentration. They did not, however, find an association with M2-like TAMs and prognosis.

In addition to predicting disease progression and risk of recurrence, analysis of macrophage populations could be useful to predict metastatic potential. A cell surface marker not specific to either M1-like, M2-like or TAM-like macrophages called sialoadhesin (also known as CD169 and siglec-1) was recently investigated for its ability to predict metastatic potential and prognosis in prostate cancer (Stromvall, Sundkvist, Ljungberg, Halin Bergstrom, & Bergh, 2017). In their study, they measured macrophage sialoadhesin expression in metastasis-free lymph nodes of 109 prostate cancer patients who had undergone surgery. They found that low expression of sialoadhesin in lymph node macrophages was associated with aggressive disease and worse prognosis. In their rodent study, they found that lymph node macrophage expression of sialoadhesin was decreased in rodents with highly metastatic prostate tumors relative to poorly metastatic tumors. Measurement of specific macrophage expression markers, rather than concentration of specific types of macrophages, could serve as valuable biomarkers for disease progression, risk of recurrence and metastatic potential.

OBESITY AND CANCER

Obesity has been associated with an increased risk of almost all cancers, including prostate cancer. The 5-year survival rate for metastatic prostate cancer is 30%, and obese men are more likely to experience metastatic prostate cancer and subsequent increased risk of death (Freedland, 2005). In men who undergo prostate biopsies, obesity is positively associated with high-grade prostate cancer (Z. Gong et al., 2006) and obesity increases the risk of dying specifically from prostate cancer (Vidal et al., 2017). These associations may be due to the increased levels of insulin, leptin, or IGF-1 in obesity, or to the presence of low-grade inflammation associated with obesity that turns down the immune system and allows the cancer to progress. IL-10 is one cytokine produced in high amounts by adipocytes that acts as an immune suppressant and may be a key factor in the link between obesity and prostate cancer progression.

Chapter 2

THESIS OBJECTIVES

IL-10 has been shown to be an important factor in both macrophage polarization and cancer progression, due to its ability to act as an immune suppressant. TGFβ is a regulatory cytokine that activates FosB to promote tumor survival, migration and invasion through induction of transcription of pro-survival genes (Barrett et al., 2017). TGFβ is also critical in maintenance of immune tolerance, another type of immune suppression (M. O. Li, Wan, Sanjabi, Robertson, & Flavell, 2006). It has also been established that IL-10 and TGFβ are secreted in high amounts by M2-like TAMs. Previous studies in our lab have found that treatment of macrophages with obese sera increases the expression of IL-10 and TGFβ, relative to macrophages treated with lean sera, implying that obese sera polarizes macrophages to a more immunosuppressive M2-like TAM phenotype (Galvan et al., 2017). Rapamycin, an inhibitor of mTOR, is a potent immune modulator that may affect how much IL-10 and TGFβ is produced by obese sera-stimulated M2-like TAMs. Rapamycin is currently approved for treatment of renal cancer, and studies are underway looking at using rapamycin for Kaposi's sarcoma, glioblastoma multiforme, and various types of lymphoma.

RESEARCH SIGNIFICANCE AND INNOVATION

If Rapamycin is shown to reduce the IL-10 and TGFβ secreted by obese-stimulated M2-like TAMs, it could be a useful treatment to block disease progression in obese prostate cancer patients placed under surveillance or to prevent recurrence after initial treatment. Reducing IL-10 and TGFβ production by obesity-stimulated M2-like TAMs could keep the immune system functioning to block cancer progression. Furthermore, identifying TAM concentration as a reliable biomarker will reduce unnecessary, aggressive treatment in patients with a low likelihood of PCa progression.

There are currently no studies on the use of Rapamycin in reducing IL-10 or TGFβ production by M2-like TAMs induced by obesity in prostate cancer patients.

MATERIALS AND METHODS

Cell lines and reagents

The androgen receptor positive prostate cancer cell lines LNCaP and LAPC-4, as well as U937 monocytes, were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). The cell lines were grown in RPMI-1640 (GIBCO Life Technologies) supplemented with penicillin, streptomycin and 10% fetal bovine serum (FBS). Androgen receptor negative prostate cancer cell line DU-145 were graciously gifted by the Tiziani lab of the University of Texas at Austin. The DU-145 were grown in EMEM (GIBCO Life Technologies) supplemented with penicillin, streptomycin and 10% fetal bovine serum (FBS). All cell lines were grown in a 5% (v/v) CO_2 humidified incubator at 37°C. Rapamycin was purchased from Sigma-Aldrich.

Serum samples

Pooled male serum was purchased from Equitech and categorized by BMI category (obese: BM I≥ 30 kg/m², non-obese: BMI < 25 kg/m²).

Conditioned media

Conditioned media (CM) was made by seeding $3x10^5$ prostate cancer cells per well in 6-well plates. Cells were serum-starved for 6 hours and then exposed to 2% obese or non-obese sera in serum-free media (SFM). The cells were exposed to the 2% sera for 1 hour, rinsed with phosphate buffered saline (PBS), then incubated in SFM. This conditioned media was collected after 24 hours and stored at -20°C for later use. The PacMetUT1 Conditioned Media was generated by Dr. Gloria Galvan in April 2017 and stored at -20°C until use.

Macrophage differentiation and polarization

At the time of seeding for experiments, U937 monocytes were matured to macrophages by adding 10.0 ng/mL tetradecanoyl phorbol acetate (TPA) (Sigma) and incubated for 48 hours (Bowers et al., 2014). Macrophages were treated with obese or non-obese sera stimulated LNCaP or LAPC4 conditioned media for 24 hours. Macrophages were treated with 10nM rapamycin or vehicle control for 30 minutes prior to treatment with Conditioned Media.

Quantitative PCR (qPCR)

RNA was extracted from treated cells using TRIzol™ Reagent (Invitrogen) following manufacturer's instructions. cDNA was generated from extracted RNA using MultiScribe™ Reverse Transcriptase, following manufacturer's instructions. Quantitative RT-PCR was performing with SYBR® Green (Life Technologies), following manufacturer's instructions for thermal cycling conditions. For quantification, relative mRNA levels were normalized to Actin. The primers used for IL-10 mRNA quantification were TCTCCGAGATGCCTTCAGCAGA (forward) and TCAGACAAGGCTTGGCAACCCA (reverse). The primers used for TGFβ mRNA quantification were TACCTGAACCCGTGTTGCTCTC (forward) and GTTGCTGAGGTATCGCCAGGAA (reverse).

Statistical Analysis

Values on graphs are presented as the mean of the three trials and the error bars are the standard deviation between the three trials. Significance was determined between the two different experimental conditions using the Student's *t* test. Significance is considered a *p* value of <0.05.

RESULTS

The results confirmed that IL-10 and TGFβ expression is increased in matured U937 cells treated with obese sera stimulated prostate cancer conditioned media, relative to the U937 cells treated with non-obese sera stimulated prostate cancer conditioned media. The results also show that treatment with 10nM rapamycin decreases the expression of IL-10 and TGFβ in U937 cells treated with obese sera stimulated prostate cancer conditioned media.

Figure 1 shows the average expression of IL-10 of three trials of treating U937 cells with non-obese sera stimulated LNCaP conditioned media, obese sera stimulated LNCaP conditioned media, and obese sera stimulated LNCaP conditioned media plus 10nM Rapamycin. Rapamycin treatment brought expression levels of IL-10 down to nearly that of the non-obese sera stimulated LNCaP conditioned media. The difference between non-obese sera stimulated LNCaP conditioned media treated U937 and obese sera stimulated LNCaP conditioned media treated U937 was significant, as was the difference between obese sera stimulated LNCaP conditioned media treated U937 and obese sera stimulated LNCaP conditioned media and 10nM Rapamycin treated U937.

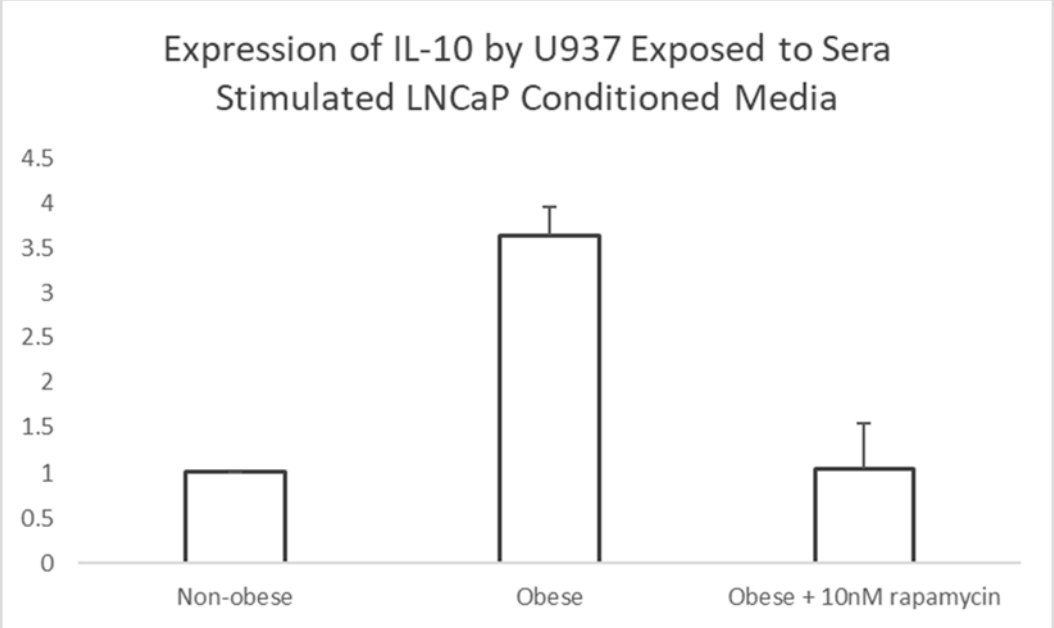


Figure 2 shows the average expression of IL-10 of three trials of treating U937 cells with non-obese sera stimulated LAPC4 conditioned media, obese sera stimulated LAPC4 conditioned media, and obese sera stimulated LAPC4 conditioned media plus 10nM rapamycin. Rapamycin treatment was not able to bring expression levels of IL-10 down to that of the non-obese sera stimulated LAPC4 conditioned media. The difference between obese sera stimulated LAPC4 conditioned media treated U937 and obese sera stimulated LAPC4 conditioned media and 10nM Rapamycin treated U937 was approaching significance.

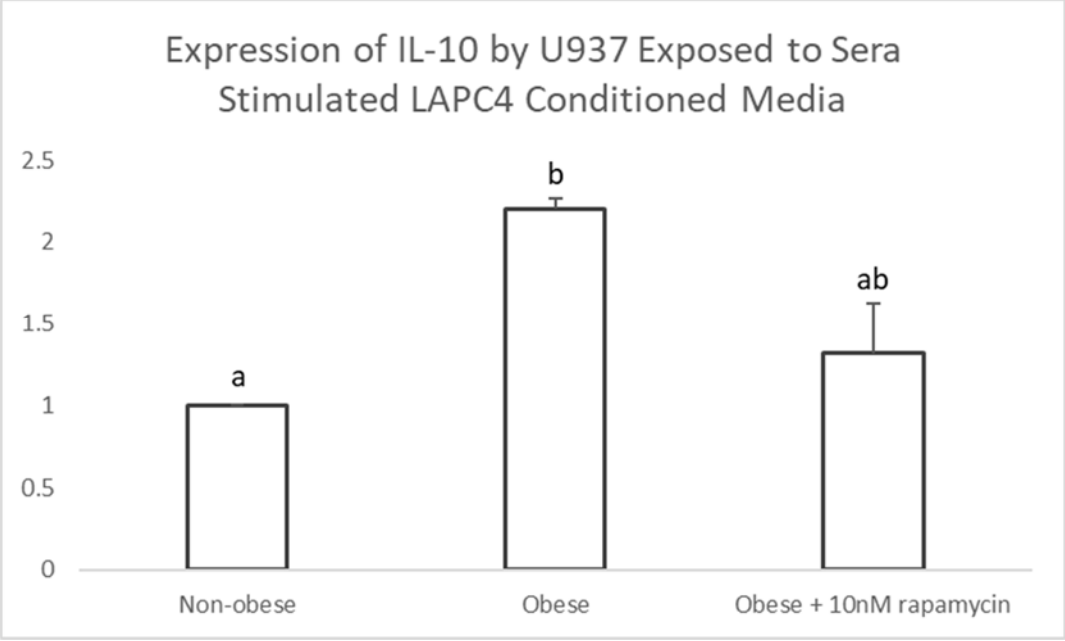


Figure 3 shows the average expression of IL-10 of three trials of treating U937 cells with non-obese sera stimulated PacMetUT1 conditioned media, obese sera stimulated PacMetUT1 conditioned media, and obese sera stimulated PacMetUT1 conditioned media plus 10nM rapamycin. Rapamycin treatment was able to reduce expression levels of IL-10 down to nearly that of the non-obese sera stimulated PacMetUT1 conditioned media. However, the differences between non-obese sera stimulated PacMetUT1 conditioned media treated U937, obese sera stimulated PacMetUT1 conditioned media treated U937 and obese sera stimulated PacMetUT1 conditioned media and 10nM Rapamycin treated U937 were not statistically significant.

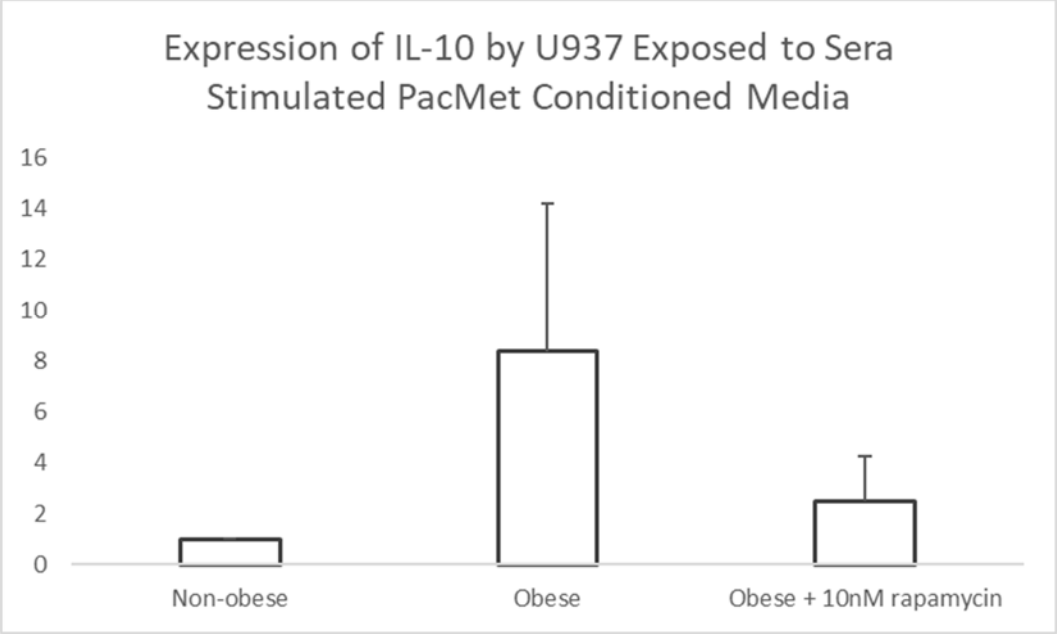


Figure 4 shows the average expression of IL-10 of three trials of treating U937 cells with non-obese sera stimulated DU-145 conditioned media, obese sera stimulated DU-145 conditioned media, and obese sera stimulated DU-145 conditioned media plus 10nM rapamycin. Rapamycin treatment brought expression levels of IL-10 down to below that of the non-obese sera stimulated DU-145 conditioned media. However, the differences between non-obese sera stimulated DU-145 conditioned media treated U937, obese sera stimulated DU-145 conditioned media treated U937 and obese sera stimulated DU-145 conditioned media and 10nM Rapamycin treated U937 were not statistically significant, though the difference between obese sera stimulated DU-145 conditioned media treated U937 and obese sera stimulated DU-145 conditioned media and 10nM Rapamycin treated U937 was approaching significance.

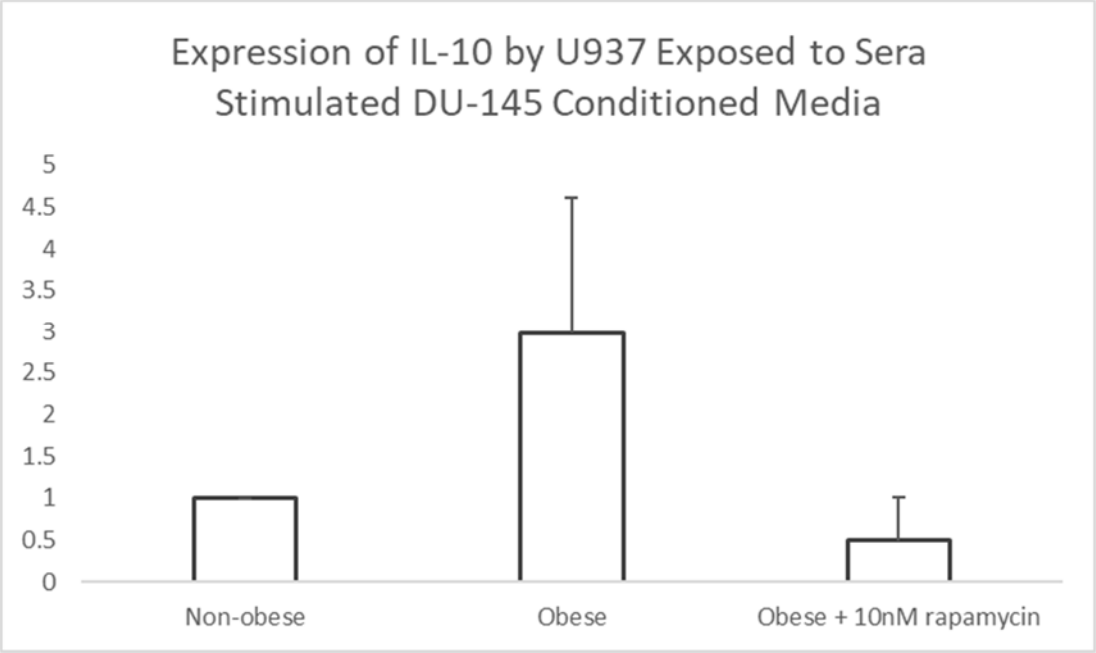


Figure 5 shows the average expression of TGFβ of three trials of treating U937 cells with non-obese sera stimulated LNCaP conditioned media, obese sera stimulated LNCaP conditioned media, and obese sera stimulated LNCaP conditioned media plus 10nM rapamycin. The differences between all three treatment conditions were statistically significant, though the Rapamycin treatment was not able to reduce TGFβ expression in the obese sera stimulated LNCaP conditioned media treated U937 down to the levels of that of the non-obese sera stimulated LNCaP conditioned media.

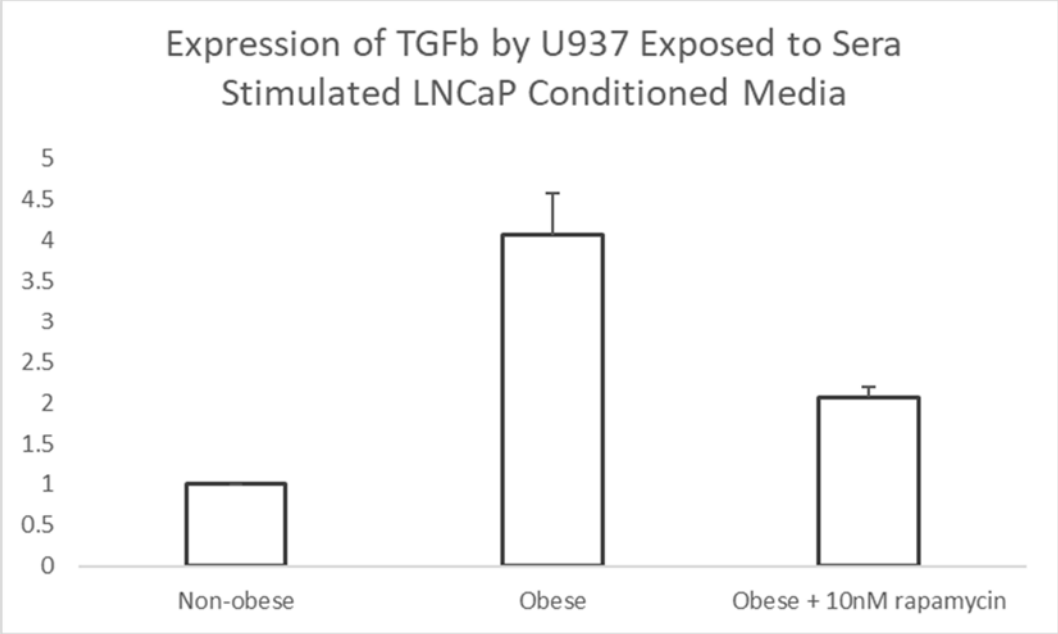


Figure 6 shows the average expression of TGFβ of three trials of treating U937 cells with non-obese sera stimulated LAPC4 conditioned media, obese sera stimulated LAPC4 conditioned media, and obese sera stimulated LAPC4 conditioned media plus 10nM rapamycin. The 10nM Rapamycin treatment reduced the expression of TGFβ in the obese sera stimulated LAPC4 conditioned media treated U937, but not as low as the level expressed by the non-obese sera stimulated LAPC4 conditioned media treated U937. The differences between the three conditions were also not statistically significant.

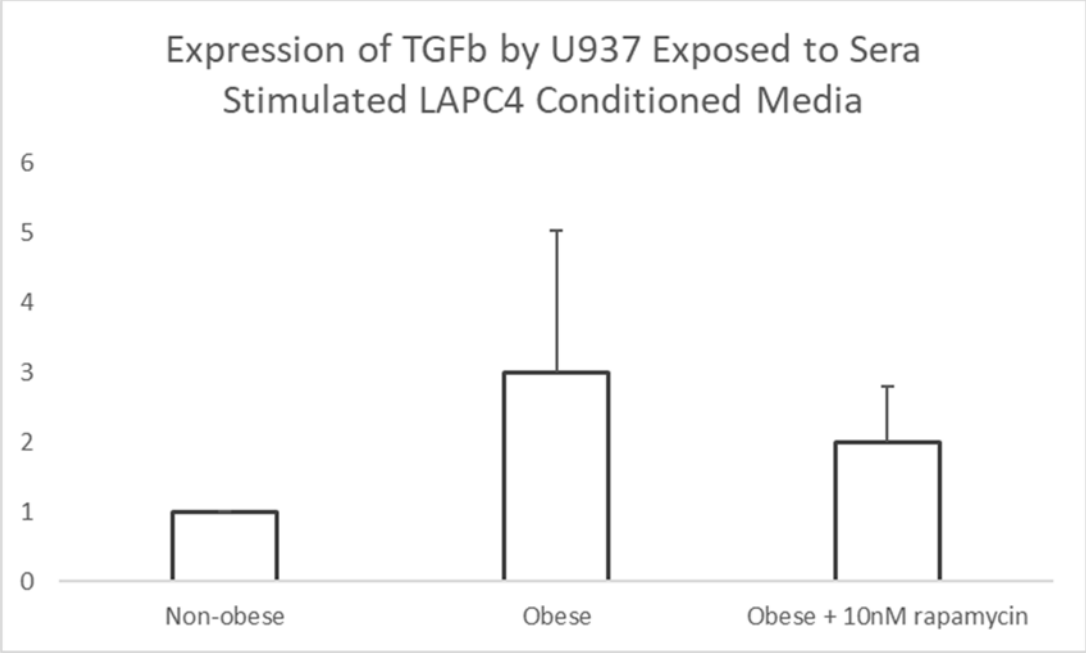


Figure 7 shows the average expression of TGFβ of three trials of treating U937 cells with non-obese sera stimulated PacMetUT1 conditioned media, obese sera stimulated PacMetUT1 conditioned media, and obese sera stimulated PacMetUT1 conditioned media plus 10nM rapamycin. The 10nM Rapamycin treatment reduced the expression of TGFβ in the obese sera stimulated PacMetUT1 conditioned media treated U937 to expression levels below that of the level expressed by the non-obese sera stimulated PacMetUT1 conditioned media treated U937. The differences between the three conditions were also not statistically significant.

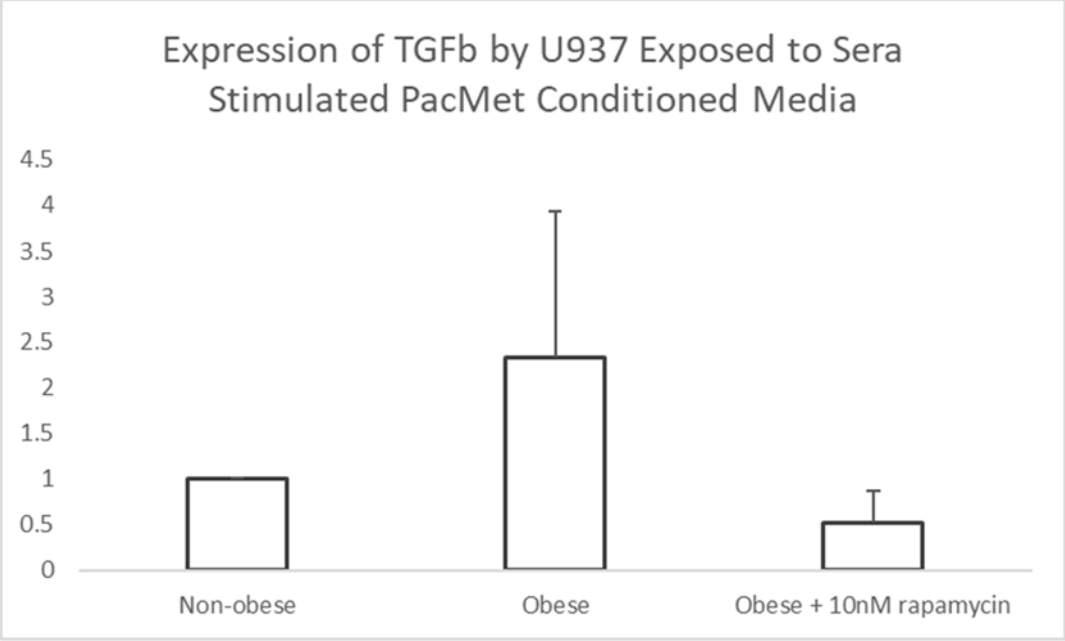
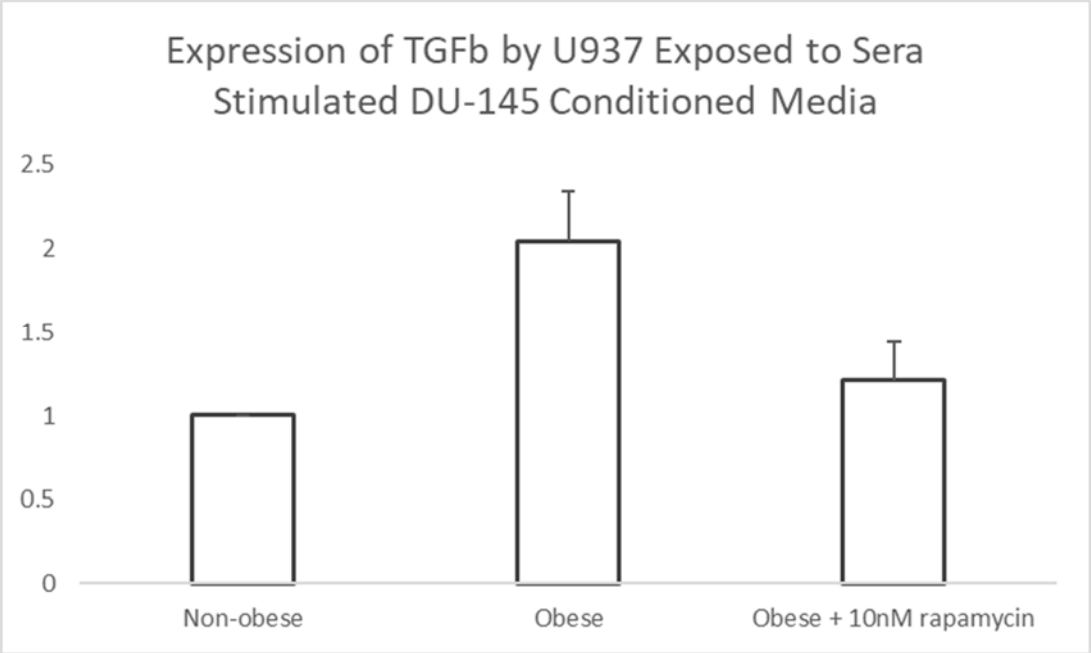


Figure 8 shows the average expression of TGFβ of three trials of treating U937 cells with non-obese sera stimulated DU-145 conditioned media, obese sera stimulated DU-145 conditioned media, and obese sera stimulated DU-145 conditioned media plus 10nM rapamycin. The 10nM Rapamycin treatment reduced the expression of TGFβ in the obese sera stimulated DU-145 conditioned media treated U937, but not as low as the level expressed by the non-obese sera stimulated DU-145 conditioned media treated U937. Only the difference between the non-obese sera stimulated DU-145 conditioned media treated U937 and the obese sera stimulated DU-145 conditioned media treated U937 was statistically significant, though the difference between obese sera stimulated DU-145 conditioned media, and obese sera stimulated DU-145 conditioned media plus 10nM Rapamycin was approaching significance.

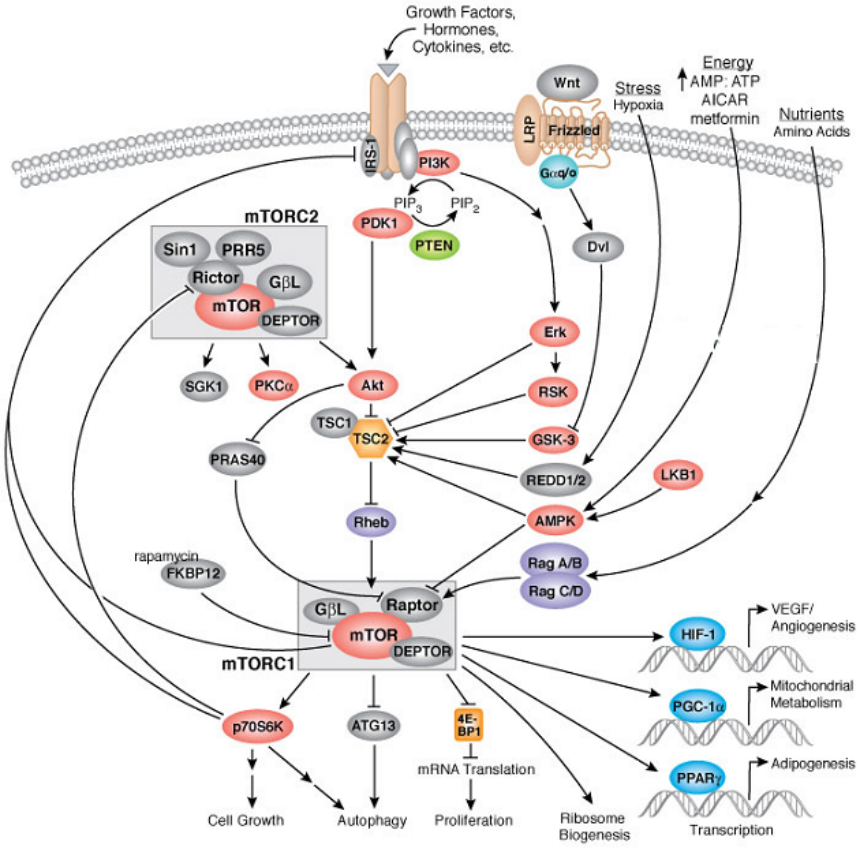


DISCUSSION

The series of experiments shown in figures 1 through 8 indicate that 10nM Rapamycin treatment reduces the expression of IL-10 and TGFβ in U937 macrophages treated with obese sera stimulated prostate cancer conditioned media. The ability for Rapamycin to reduce expression of IL-10 and TGFβ in U937 was observed independently of androgen receptor status. LNCaP, LAPC4 and PacMetUT1 cells are all androgen receptor sensitive, while DU-145 cancer cells are androgen receptor independent. The LNCaP conditioned media treated cells displayed the most statistically significant data concerning expression of IL-10 and TGFβ.

The mechanism by which the Rapamycin treatment reduces the expression of IL-10 and TGFβ likely does not involve the androgen receptor, but likely does involve other transcription factors. The mechanism relies heavily on understanding the pathways that are affected by Rapamycin and other mTOR inhibitors. mTOR inhibitors such as Rapamycin work by binding directly to the mTOR complex 1 (mTORC1), which affects cell growth, autophagy, translation, proliferation, and several transcription factors including HIF-1, PGC-1α, PPARγ and STAT3. mTORC1 plays a role in cancer development, as it functions as a nutrient sensor activating or blocking translation of proteins that allow cells to grow and proliferate. mTORC1 is also a key regulator of autophagy, the degradation of cells with the subsequent recycling of their components (Zoncu, Efeyan, & Sabatini, 2011). There are multiple pathways that lead to activation of mTORC1 under nutrient-rich conditions, or to repression in times of low energy or nutrient status. In nutrient-rich conditions (such as obesity), IGF-1 can activate mTORC1 through the PI3K-Akt pathway and the MAP/ERK pathway, both of which block hydrolysis of Rheb-GTP, which in turn keeps mTORC1 active. Rheb-GTP is typically hydrolyzed by the TSC1/TSC2 heterodimer, which is dissolved through activation of both the PI3K-Akt pathway and the MAP/ERK pathway. The

TSC1/TSC2 heterodimer is also dissolved by activation of the Wnt pathway, as well as by cytokines such as TNFα. Keeping the TSC1/TSC2 heterodimer active and capable of hydrolyzing Rheb-GTP is a key player in maintaining responsive and controlled mTORC1 function. In nutrient-rich conditions, active mTORC1 acts directly on p70-S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E (eIF4E). S6K1 and eIF4E regulate translation by meeting at the 5' end of an mRNA. In nutrient-poor conditions, mTORC1 is typically inactive and promoting autophagy. This is one of the ways in which obesity is thought to promote cancer progression.



https://agscientific.com/blog/2019/05/rapamycin-faqs/

Targeting mTORC1 has therapeutic benefit to cancer because when it's active, cells are proliferating and surviving, and when it's inactive, autophagy and cell cycle reprogramming occurs as necessary (Fingar et al., 2004). Inducing low energy or nutrient status is a broadly researched method to reduce mTORC1 activity. Calorie restriction reduces mTORC1 activity by

several mechanisms, including reduction of ATP and IGF-1 (Hursting, Lavigne, Berrigan, Perkins, & Barrett, 2003). A reduction in ATP activates the AMPK pathway, an opposing pathway and negative regulator to the mTOR proliferation pathway. Activation of the AMPK pathway upregulates the formation of the TSC1/TSC2 heterodimer complex that can hydrolyze Rheb-GTP and thus inactivate mTORC1. Deprivation of growth signals such as IGF-1 also activates the TSC1/TSC2 heterodimer, inhibiting mTOR activation stimulated by Rheb-GTP (Inoki, Li, Xu, & Guan, 2003). While in vitro studies have shown that calorie restriction reduces IGF-1 levels, human studies have thus far been inconclusive and may suggest that protein restriction or cycling, rather than calorie restriction is the key to reducing IGF-1 levels (Fontana et al., 2016; Fontana, Weiss, Villareal, Klein, & Holloszy, 2008; Parrella et al., 2013).

While calorie or protein restriction, intermittent fasting or ketogenic diets may be effective at downregulating mTORC1 to improve therapeutic response, prescribing medication may improve patient compliance even further, especially in cancer patients who may already be struggling with appetite and weight loss. Rapamycin is a calorie restriction mimetic currently thought to have beneficial cancer-fighting properties due to its ability to inhibit mTORC1.

Rapamycin was originally discovered as an immunosuppressant for use in transplant patients. It is the original antiproliferative mTOR inhibitor. Rapamycin inhibits mTORC1 by binding directly to the complex after first complexing with FK-binding protein 12 (Mukherjee & Mukherjee, 2009). By inhibiting mTORC1, rapamycin slows or stops proliferation of tumor cells and upregulates tumor cell apoptosis. By the same mechanism, rapamycin acts as an immunosuppressant by inhibiting proliferation of immune B and T cells (Law, 2005). Due to its strong immunosuppressant activity and powerful upregulation of autophagy in all cell types including healthy cells, rapamycin is more effective in conjunction with other therapies that can

balance the annihilation of healthy cells. Rapamycin is currently being evaluated in clinical trials as a supplementary drug to improve the efficacy of hormone therapy, angiogenesis inhibitors, inhibitors of the IGF-1 receptor and therapy targeting receptor tyrosine kinase (RTK) targeted therapy in many different types of cancer.

While most of the current research looks at the effects of Rapamycin on cellular metabolism, proliferation and protein translation, evidence is showing that Rapamycin also has useful effects on the immune system and transcription. Transcription factors mTORC1 is known to play a hand in regulating include STAT3, SREBPs, PPARγ, PPARα, HIF1α, YY1–PGC1α and TFEB (Laplante & Sabatini, 2013). The mechanism by which Rapamycin reduces the expression of IL-10 and TGFβ in U937 cells treated with obese sera stimulated prostate cancer conditioned media likely involves STAT3. A study from 2018 found that conditioned media from cancer cells induced macrophages towards an M2 phenotype and greater expression of IL-10 through increased phosphorylation of STAT3 (Solis-Martinez et al., 2018). There are currently no studies directly linking STAT3 with TGFβ expression, but as STAT3 is involved in transcribing a large variety of genes related to cell growth and apoptosis, it is not out of the realm of possibility that it helps transcribe the genes for TGFβ (Yuan et al., 2004).

Studies have examined how mTOR regulates transcription factors including STAT3. To become active as transcription factor, STAT3 must be phosphorylated at either Tyr705 or Ser727 (Decker & Kovarik, 2000). mTOR is a kinase able to phosphorylate STAT3 at Ser727, and Rapamycin inhibits mTOR from this phosphorylation and activation of STAT3 (J. H. Kim, Yoon, & Chen, 2009). A review by Laplante, et al., 2013 determined that the mTORC1-STAT3 pathway plays a significant role in cancer progression and should be considered a therapeutic target (Laplante & Sabatini, 2013). The effect Rapamycin has on the mTORC1-STAT3 axis is a likely

mechanism to explain the observed decrease in expression levels of IL-10 and TGFβ in the obese sera stimulated prostate cancer M2-like U937 TAMs.

There are a few other alternative mechanisms by which Rapamycin may reduce the expression of IL-10 and TGFβ in M2-like tumor-associated U937 macrophages treated with obese sera stimulated prostate cancer conditioned media. Inhibiting mTOR in U937 macrophages may affect the initial polarization of the macrophages. As the Rapamycin was treated prior to polarization with conditioned media, inhibiting mTOR may reduce the effect the conditioned media has on polarizing the macrophages towards the M2-like TAM phenotype. Fewer U937 macrophages undergoing initial polarization to the M2-like TAM phenotype, and a subsequent decrease in the percentage of macrophages polarized to the M2-like TAM phenotype would explain the decreased IL-10 and TGFβ expression seen in the RT-qPCR results. A second possibility is that mTOR inhibition is targeting the M2-like TAMs, and only the M2-like TAMs, for apoptosis. M2-like TAMs primarily undergoing apoptosis and a subsequent decrease in the percentage of macrophages polarized to the M2-like TAM phenotype would explain the decreased expression levels of IL-10 and TGFβ in the RT-qPCR results.

Chapter 3

FUTURE DIRECTIONS

An important future direction is to determine if inhibition of mTOR by Rapamycin in obese sera stimulated prostate cancer conditioned media treated M2-like U937 TAMs is just affecting transcription of IL-10 and TGFβ, or if the Rapamycin is possibly promoting apoptosis in only the M2-like TAMs or blocking initial polarization to the M2-like TAM phenotype. To answer this question, a series of Western blot and flow cytometry experiments could be performed.

Western blots look at the phosphorylation and subsequent activation of STAT3 transcription factor. Comparing the phosphorylated STAT3 in lean sera stimulated prostate cancer conditioned media treated U937 macrophages, obese sera stimulated prostate cancer conditioned media treated U937 macrophages and obese sera stimulated prostate cancer conditioned media treated U937 macrophages also treated with 10nM Rapamycin could determine if the decreased expression levels of IL-10 and TGFβ is due to blocking phosphorylation of STAT3.

Annexin-V/propidium iodide (PI) flow cytometry is the most useful tool to determine if mTOR inhibition is selectively inducing apoptosis in the obesity-induced M2-like TAMs. Flow cytometry can also be used to determine if the Rapamycin treatment is affecting the initial polarization to the M2-like TAM phenotype. To conduct the flow cytometry experiments, macrophages can be polarized using the sera stimulated prostate cancer conditioned media, then treated with Rapamycin. All macrophages and specific M2-like TAMs can then be tagged with antibodies – F4/80 for total macrophage content, CD206 for M2-like TAMs, and CD86 for M1-like macrophages. The macrophages can also be tagged with fluorophores to Annexin-V and PI, markers that determine viability of cells and whether they are undergoing apoptosis.

Another direction is to examine the effect reducing IL-10 and TGFβ expression in obese-sera stimulated M2-like TAMs has on other immune cells of the TME, such as T cells. Cells of the immune system function in tandem to fight cancer and activation of one immune cell may affect the activation of other immune cells. One such concurrent study is our lab looks at how obesity-induced M2-like TAMs affects CD4+ T cell activity. This study found that the obesity-induced M2-like TAMs increased expression of IL-6 by CD4+ T cells, implying suppressed normal activation. As discussed earlier, adipocytes also produce elevated IL-6, which interacts with cancer cells that in turn preferentially polarized macrophages towards the M2-like TAM phenotype. This effect can be viewed as a feedback cycle where macrophages and T cells continually interact to suppress their cancer fighting abilities. A future direction could be to treat CD4+ T cells with conditioned media from obesity-induced M2-like TAMs treated with Rapamycin to determine in the reduced IL-10 and TGFβ effects the subsequent expression of IL-6 by CD4+ T cells.

Another future direction is to examine the associations between M2-like TAM concentration and obesity and prostate cancer recurrence. Human studies have demonstrated a correlation between Gleason score and local tumor M2-like TAM concentration. Previous studies in the rodent PTEN KO prostate cancer model have mirrored the findings in the human studies, as well as finding an association between obesity and local M2-like TAM concentration. C57BL/6J ARR2PB-CreER(T2)xPten$^{fl/fl}$ mice (n=13) were randomized at 3 weeks of age to either a high fat diet (60% kcal from fat) to induce obesity or a control diet (10% kcal from fat), which they were fed ad libitum for 17 weeks. At 6 weeks of age, 4-hydroxy tamoxifen (OHT) was administered to half of the mice to induce a prostate-specific PTEN deletion. Tissue from all mice was isolated, formalin-fixed and paraffin-embedded for pathology and biomarker evaluation. Prostates from the obese mice had higher total macrophage (F4/80+) and M2-like TAM (CD206+) concentration

than non-obese mice when comparing those with high-grade tumors only. Samples from patients with low-grade disease choosing surveillance could be evaluated to determine if 1) M2-like TAMs concentration is higher in obese patients and 2) if M2-like TAM concentration predicts disease recurrence.

Immunohistochemistry could be used to evaluate M2-like TAM concentration as a possible biomarker. Samples from 32 normal weight and overweight prostate cancer patients with similar Gleason scores could be analyzed to determine 1) if M2-like TAM concentration is higher in obese patients and 2) if M2-like TAM concentration predicts disease recurrence. The Mays Cancer Center at UT Health San Antonio can provide de-identified slides with no information regarding weight or disease recurrence. Human prostate tumor samples could be fixed in 10% formalin and embedded in paraffin. One of the three sectioned slides from each patient can be stained with hematoxylin and eosin (H&E) to identify the type of tissue and the different tissue components. The H&E slide confirms that the prostate sample is cancerous, and it can be used to compare with the antibody stained slides. The F4/80 antigen can be used to identify total macrophage concentration in the sectioned slide. The CD206 antigen can be used to identify M2-like TAMs in the sectioned slide. Undergraduate volunteers can be trained to count the macrophages and M2-like TAMs in the stained slides, and a minimum of three different people should count the macrophages and M2-like TAMs on each slide. The average of the three+ counts can be taken to calculate the ratio of M2-like TAMs to total macrophages. The findings could then be relayed back to the Mays Cancer Center at UT Health San Antonio, upon which the information can be combined to determine the associations between obesity, M2-like TAM concentration and disease recurrence.

CONCLUSION

With PCa being the second most commonly diagnosed cancer type in men and the large baby boomer population reaching the ages when PCa is most commonly detected, there is a strong need for better treatments and prognostic biomarkers for PCa. As PCa treatments differ based on disease stage, discerning which PCa patients with more progressive cancers warrant more aggressive treatments will aid in improving outcomes. Use of reliable prognostic biomarkers are an important way of discerning which cancers are at risk of progressing, recurring or metastasizing. Since TAMs are typically associated with more aggressive, higher-grade PCa, they may serve as a promising prognostic biomarker. TAMs have profound effects on angiogenesis, metastasis, tumor growth and survival and the EMT pathway, and thus they may also serve as a potential therapeutic target to slow cancer progress. TAM-targeting therapies are showing promise in pre-clinical and clinical studies. Research into blocking macrophage infiltration, inhibiting chemokines that induce TAM polarization, and repressing angiogenic and metastatic functions of TAMs have all produced positive results. However, further research is still needed to determine if these therapies can be used alone or are more effective in combination with immunotherapy or chemotherapy.

This study shows that Rapamycin treatment can reduce IL-10 and TGFβ expression from obesity-induced M2-like tumor-associated macrophages. Reduced IL-10 and TGFβ expression may serve to activate the immune system, allowing a patient's immune system to slow the cancer progression.

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